U.S. DEPARTMENT OF COMMERCE
National Technical Information Service
PB-285 002

Brominated Compounds Found in Waste-Treatment Effluents and Their Capacity to Bioaccumulate

Environmental Research Lab -Duluth, Minn

1978

PB 285 002

WATER CHLORINATION
Environmental Impact and Health Effects

Volume 2

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U. S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

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230 Collingwood, P. O. Box 1425, Ann Arbor, Michigan 48106

Library of Congress Catalog Card No. 77-92588
ISBN 0-250-40201-7

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TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. RECIPIENT'S ACCESSION NO.
EPA-600/J-78-036	JOURNAL ARTICLE	PB285002

4. TITLE AND SUBTITLE	5. REPORT DATE
Brominated Compounds Found in Waste-Treatment Effluents and Their Capacity to Bioaccumulate	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) Douglas W. Kuehl, Gilman D. Veith, and Edward N. Leonard	8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS U.S. Environmental Protection Agency Environmental Research Laboratory-Duluth 6201 Congdon Boulevard Duluth, Minnesota 55804	10. PROGRAM ELEMENT NO. 1BA608 11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS Same	13. TYPE OF REPORT AND PERIOD COVERED 14. SPONSORING AGENCY CODE EPA-600/03

15. SUPPLEMENTARY NOTES REFERENCE: Kuehl, Douglas W., Gilman D. Veith, and Edward N. Leonard. 1978. Brominated Compounds Found in Waste-Treatment Effluents and Their Capacity to Bioaccumulate. pp. 175-192. In: WATER CHLORINATION, Environmental Impact and Health Effects, Volume 2. R. L. Jolley (ed.). Ann Arbor Science Publishers, Inc., Ann Arbor, Michigan.

16. ABSTRACT

Gas chromatography-mass spectrometry was used to analyze residues in fathead minnows (Pimephales promelas) which were exposed to non-disinfected and bromine chloride disinfected wastewater effluent. A series of brominated organic chemicals was identified in the fish exposed to the bromine chloride disinfected effluent but were not detected in fish exposed to the non-disinfected wastewater. Two mechanisms for the presence of these residues include the aqueous bromination of precursors followed by bioaccumulation of the products, and the bromination of precursors by the fish.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Chlorination Water treatment Fish residues Waste water	Organic chemistry Chlorination	07C

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	22
	20. SECURITY CLASS *(This page)*	22. PRICE
	UNCLASSIFIED	A02-A01

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

EPA-600/J-78-036
JOURNAL ARTICLE

14

BROMINATED COMPOUNDS FOUND IN WASTE-TREATMENT EFFLUENTS AND THEIR CAPACITY TO BIOACCUMULATE

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INTRODUCTION

At the 1975 Conference on The Environmental Impact of Water Chlorination, this laboratory presented the results of analyses of organochlorine residues in fathead minnows (*Pimephales promelas*) exposed to nondisinfected and chlorinated wastewater effluent at an experimental sewage treatment plant in Grandville, Michigan.[1] At this conference we present the results of further residue studies on samples from the same Grandville plant and an additional plant at Wyoming, Michigan, and will emphasize brominated compounds. Methodology developed for the isolation and identification of brominated organic compounds from tissue samples will be described and the bioaccumulation of several identified brominated compounds will be discussed.

A complete description of the Grandville, Michigan, experimental sewage treatment project has been described by Ward

et al.[2] Briefly, at both the Grandville and Wyoming sites, nondisinfected activated sludge effluent was split, diluted and treated with disinfectant. The resulting wastewater was used for continuous-flow chronic toxicity tests using fathead minnows. Minnows exposed to bromine-chloride-treated and nondisinfected effluent were subsequently analyzed for this study.

BACKGROUND TO TISSUE PREPARATION METHODOLOGY DEVELOPMENT

The analysis of halogenated polar organic compounds from tissue samples has been previously accomplished using absorption chromatography with florisil for sample cleanup, and gas chromatography using an electron capture detector (EC/GC) for identification. This methodology is adequate when analyzing for specific compounds and when standards are available. If exploratory work is being done it is essential to use gas chromatography-mass spectrometry (GC/MS). Often, tissue sample preparation methodology developed for analysis by EC/GC cannot be used when the mass spectrometer is the GC detector because of spectral interferences due to the coelution of lipids from the adsorption columns. Gel permeation chromatography (GPC), however, has been shown to be a highly efficient technique for the cleanup of fatty samples for GC/MS analysis[3] of both nonpolar[4] and polar[5] compounds. Therefore, a two-step GPC cleanup system was developed for these analyses that provided rapid and efficient isolation of polar halogenated organics from tissue.

The isolation of nonpolar compounds such as polychlorinated biphenyls (PCBs) from fish lipids can be accomplished at nearly 100% yield when using BIO-RAD SX-2 [copoly(styrene-2% divinylbenzene)] gel beads and cyclohexane (C_6H_{12}) as the solvent,[3] while the recovery of polar compounds, such as pentachlorophenol, was generally less than 10%. With this system, a mixture of equal quantities of corn oil, Aroclor 1254 (PCB) and pentachlorophenol (PCP) could be completely separated. When the more polar solvent, methylene chloride, is used, high recoveries of both the PCB mixtures and PCP can be obtained; however,

this system did not separate a mixture of PCB and PCP. A 1:1 mixture of cylcohexane and methylene chloride on BIO-RAD SX-2 was found to provide excellent separation of the corn oil, PCB and PCP mixture at 100% yields of each component. Figure 1 shows a GPC chromatogram showing the separation of corn oil, PCB, tribromophenol and *p*-bromophenol on this system. Table I lists the results of a retention volume and recovery study completed on selected brominated organic compounds in order of retention volume shift going from the 100% CH_2Cl_2 system to the 1:1 C_6H_{12}/CH_2Cl_2 solvent system.

The overall sample preparation procedure then involves three steps. First, the tissue is extracted with an organic solvent to obtain the fish oil. Second, the lipid material is separated from the low-molecular-weight compounds (LMWC) on the SX-2/CH_2Cl_2 GPC system and the LMWCs are fractionated on the SX-2-/CH_2Cl_2/C_6H_2 GPC system and, third, the collected fractions are analyzed by GC/MS.

EXPERIMENTAL

Procedure

Blend 100 g ground fathead minnow (*Pimephales promelas*) tissue with enough anhydrous sodium sulfate to dry sample. Extract with hexane/acetone (1+1) in Soxhlet extractor for 8 hr. Evaporate solvent and dilute concentrated oil to 100 mg/ml with CH_2Cl_2. Inject a series of 5-ml aliquots on GPC/CH_2Cl_2 system, operating at 3.5 ml/min. Collect proper fraction (Figure 2, upper trace) in a Kuderna-Danish apparatus. Concentrate sample to 5 ml.

Equilibrate GPC system by recycling CH_2Cl_2/cyclohexane. Inject 5-ml sample and collect fractions (Figure 2, lower trace).

Apparatus and Reagents

Solvents: Hexane, cyclohexane, methylene chloride and acetone, redistilled pesticide grade (Burdick and Jackson).

Gel Permeation System: Dual 25-cm x 2.5-cm glass columns connected in series and filled with 100-200 mesh BIO-RAD

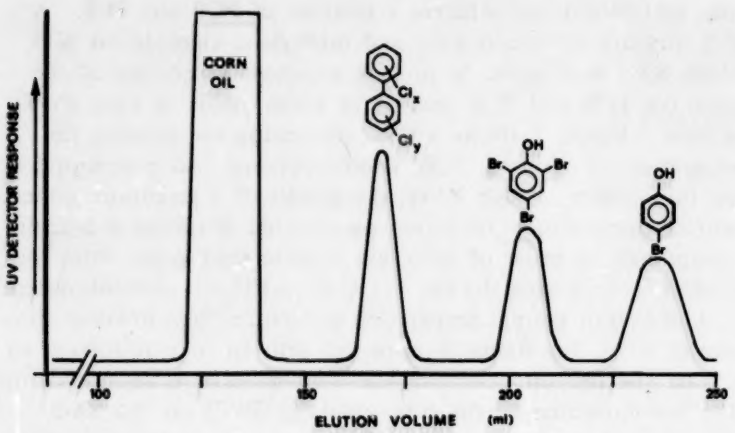

Figure 1. Gel permeation chromatogram of a mixture of corn oil, Aroclor 1254, 2,4,6-tribromophenol and *p*-bromophenol on BIO-RAD SX-2 beads. The solvent is a mixture of cyclohexane and methylene chloride.

Table I. Gel Permeation Chromatography Retention Volume and Recovery Data for Selected Standards

	CH_2Cl_2		Cyclohexane/CH_2Cl_2		
Compound	Retention Volume	Percent Recovery	Retention Volume	Percent Recovery	Retention Volume Shift
Aroclor 1254	160-190	100	160-190	100	0
p-Bromoanisole	164-172	95	176-208	60	12
2,4-Dibromoanisole	162-169	84	178-222	67	16
o-Bromophenol	166-182	98	184-208	84	18
2,4-Dibromophenol	162-183	94	182-211	100	20
2,4,6-Tribromoanisole	166-185	89	190-210	98	24
Pentachloranisole	156-174	100	174-200	75	16
2,4,6-Tribromophenol	168-160	100	194-212	80	50
5-Bromoindole	167-182	100	244-270	61	58
p-Bromophenol	164-186	100	220-244	94	66
Phenanthrene	168-195	99	250-284	87	82

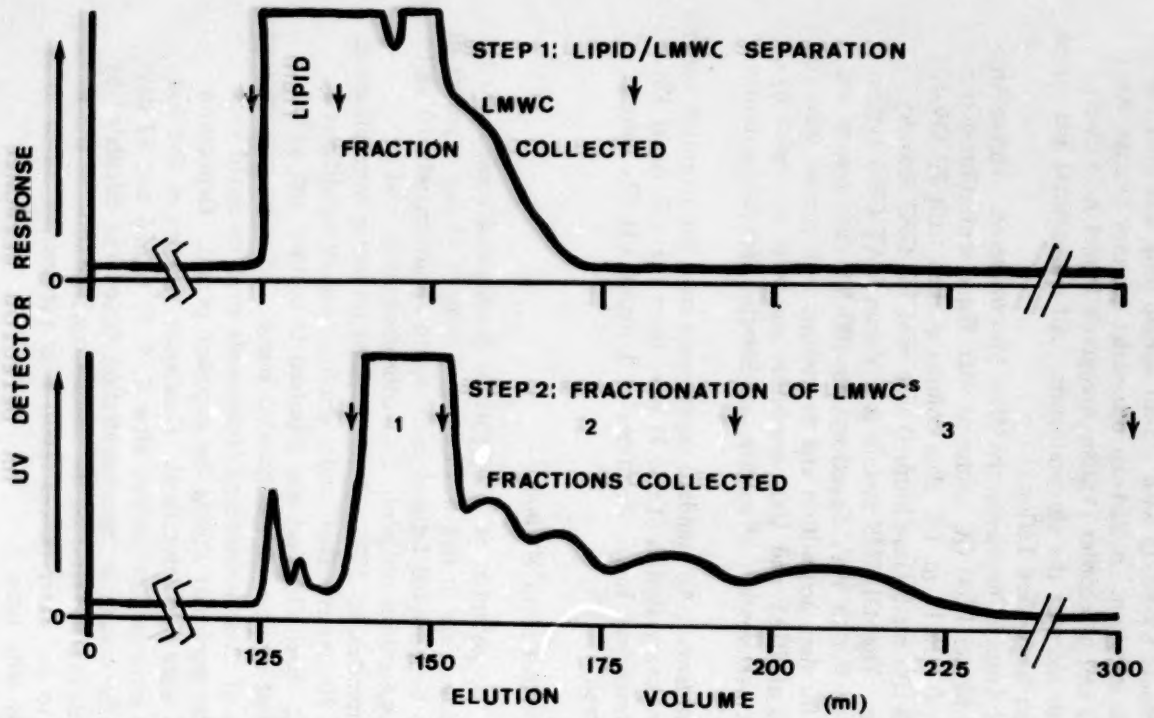

Figure 2. Gel permeation chromatograms of lipid/LMWO separation (top trace) and fractionation of LMWOs (lower trace).

SX-2 beads. A high-pressure all-Teflon® sample valve (Cheminert Model SV-8031) with a 5-ml sample loop was used for sample injection. A 254-nm ultraviolet detector (Varian Areograph) and a recorder (Varian Areograph Model A-25) were used to monitor the chromatogram. All connections and sample transfer lines were Teflon.

Gas-Liquid Chromatograph/Mass Spectrometer: Varian Areograph Model 1700 GC equipped with flame ionization detector and 6-ft x 0.11-in. i.d. glass column packed with 3% OV-101 on 80-100 mesh Gas-Chrom Q was used for GPC recovery studies. The GLC/MS system is a Varian MAT CH-5 system, and the Varian MAT Spectrosystem-100 MS data system was used for data acquisition and processing. All spectra were obtained at 70-eV and 1000 resolution and were processed by direct examination of spectra and Selected Ion Summation (SIS).[6]

Standards: All standard compounds used for retention volume and recovery studies (Table I) were from the Lab Assist Kit (Chemservices, Inc.). Solutions of 1 mg/ml CH_2Cl_2 were prepared.

Bioconcentration Method

The potential of the identified brominated compounds to bioaccumulate in fish was evaluated using a 32-day exposure of 4- to 6-month-old fathead minnows to approximately 10 μg/l of 2,4,6-tribromophenol, 2,4,6-tribromoanisole, and 5-bromoindole at 25°C. Thirty fathead minnows were placed in each 30-liter aquarium under continuous-flow conditions at 25°C. Each chemical was dissolved in acetone and added to the test aquaria with a metering pump. Aqueous concentrations of these brominated compounds were measured each regular work day during the exposure period. Depuration rates were not determined. Composite samples of five fish were removed for analysis after 2, 4, 8, 16, 24 and 32 days, and the maximum bioconcentration factor was calculated by calculating the ratio of the residue concentration in the whole fish to the water concentration after the residue ceased to increase with time.

RESULTS

A comparative study of fish tissue residues from nondisinfected and bromine chloride-disinfected sewage effluent has resulted in the isolation and identification of a series of brominated phenols, anisoles and heterocyclic aromatics at both the Grandville and Wyoming sites. Table II lists the results of the Grandville study and Table III lists the results of the Wyoming study. In each case brominated compounds were observed only in fish exposed to bromine chloride-disinfected sewage effluent and not in the fish exposed to the nondisinfected effluent.

Table II. Brominated Compounds Identified in Fish Samples from the Experimental Sewage Treatment Plant at Grandville, Michigan

	Exposure			
	Bromine Chloride		Nondisinfected	
Compound	Identification Method[b]	Concentration[a] Estimation (ng/g)	Identification Method[b]	Concentration[a] Estimation (ng/g)
Bromoindole	GC,MS	100-200	ND	-
Dibromoindole	MS	10-50	ND	-
Tribromoindole	MS	10-50	ND	-
Tetrabromoindole	MS	10-50	ND	-
Dibromomethylindole	MS	10-50	ND	-
Tribromomethylindole	MS	10-50	ND	-
Tetrabromomethylindole	MS	10-50	ND	-
Dibromomethylbenzthiazole	MS	5-25	ND	-
Dibromophenol	MS	10-50	ND	-
Tribromophenol	GC,MS	50-100	ND	-
Chlorodibromophenol	MS	5-25	ND	-
Dichlorobromophenol	MS	5-25	ND	-
Dibromomethylphenol	MS	5-25	ND	-
Dibromoanisole	MS	10-50	ND	-
Tribromoanisole	GC,MS	50-100	ND	-

[a]Based on pentachloroanisole ion intensity mass spectral data.
[b]GC = retention time correlation; MS = spectral comparison; ND = not detected.

Table III. Brominated Compounds Identified in Fish from the Experimental Sewage Treatment Plant at Wyoming, Michigan

	Exposure			
	Bromine Chloride		Nondisinfected	
Compound	Identification Method[b]	Concentration[a] Estimation (ng/g)	Identification Method[b]	Concentration[a] Estimation (ng/g)
Bromoindole	GC,MS	100-200	ND	-
Dibromoindole	MS	10-50	ND	-
Dibromomethylindole	MS	10-50	ND	-
Tribromoanisole	GC,MS	50-100	ND	-
Tribromophenol	GC,MS	50-100	ND	-

[a]Based on mass spectral data.
[b]GC = retention time correlation; MS = spectral comparison; ND = not detected.

The predominant heterocyclic aromatic in both studies is bromoindole at a concentration estimate of 100-200 ppb. In addition, isomers of di-, tri- and tetrabromoindoles were observed in the Grandville study although at lower concentration estimates of 5-50 ppb. The predominant phenol was tribromophenol (TBP) which, based on GC-retention-time data correlations, was probably 2,4,6-tribromophenol. TBP was estimated to be in a concentration range of 50-100 ppb. The predominant anisole was also tribrominated and, again based on retention-time data correlations, was probably 2,4,6-tribromoanisole (TBA). TBA concentration range was also estimated to be 50-100 ppb.

Figure 3 shows a GPC chromatogram of the fractionation of the LMWCs isolated from the fish exposed to the bromine chloride effluent from the Wyoming study. As can be seen, pentachloroanisole (PCA) and phenanthrene, which are contaminants throughout the entire bioassay system and thus presumed to be in the raw waste, elute in Fractions 2 and 3, respectively. Those compounds, therefore, can serve as internal standards to

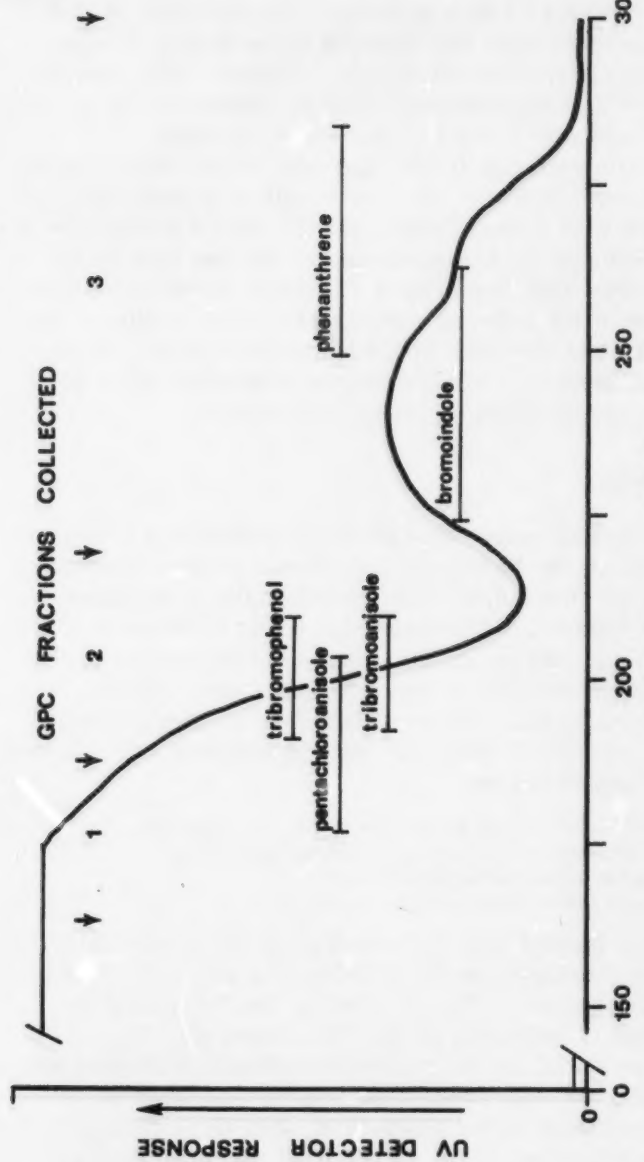

Figure 3. Gel permeation chromatogram of fish oil extracted from fathead minnows exposed to bromine chloride disinfected wastewater. The retention volume of selected organic chemicals is indicated by the horizontal bars.

determine if equal quantities of samples are being analyzed for each GPC fraction of each exposure. The SIS plots of PCA and phenanthrene from the Wyoming study (Figure 4) show that this situation does indeed exist. Figures 5 and 6 are examples of SIS plots comparing levels of brominated compounds in GPC Fractions 2 and 3 of the Wyoming study.

The bioconcentration factors, the ratio of the concentration of the chemical in fish to the concentration in water after the fish residue is at a steady state, were 83 for 2,4,6-tribromophenol; 1300 for 2,3,6-tribromoanisole; and less than 10 for 5-bromoindole. The data indicate that these chemicals do bioaccumulate in the order of their lipophilicity, though not nearly to the extent of the more lipophilic compounds such as polychlorinated biphenyls, which bioaccumulate over 60,000 times the water concentration under these conditions.

DISCUSSION

Disinfection of sewage with bromine chloride as an alternate to chlorination has been evaluated because of the bactericidal properties of bromamines[7] and the instability of bromamines, which has resulted in decreased fish toxicity.[8] However, few data presently exist concerning organobromine residues in fish exposed to disinfection of sewage with bromine chloride. The data reported in this study must therefore be examined for possible explanations to account for the observed data. Several possible explanations are:

1. bromination of precursors as a result of disinfection,
2. contamination of the effluent before disinfection,
3. the use of contaminated food, and
4. contamination from dilution by well water.

In addition, point 1 may be derived from either or both of two routes: the aqueous bromination of precursors followed by bioaccumulation of the products by the fish and/or the bromination of precursors by the fish themselves.

If the source of the brominated compounds in the fish was the contaminated fish food, contaminated well water or contaminated effluent before disinfection, then these chemicals should be a constant factor to which all fish were exposed.

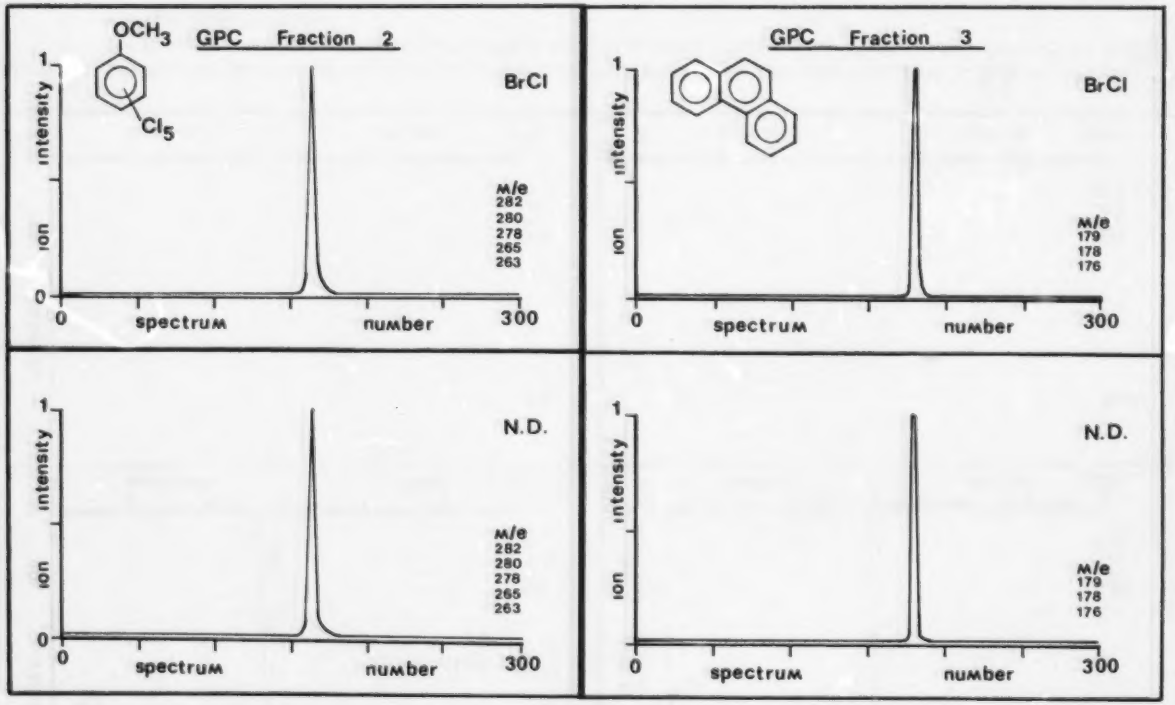

Figure 4. Selected ion summation chromatogram searching for pentachloroanisole and phenanthrene in fathead minnows from the Wyoming, Michigan, experimental sewage treatment plant. BrCl means bromine chloride-treated. N.D. means nondisinfected.

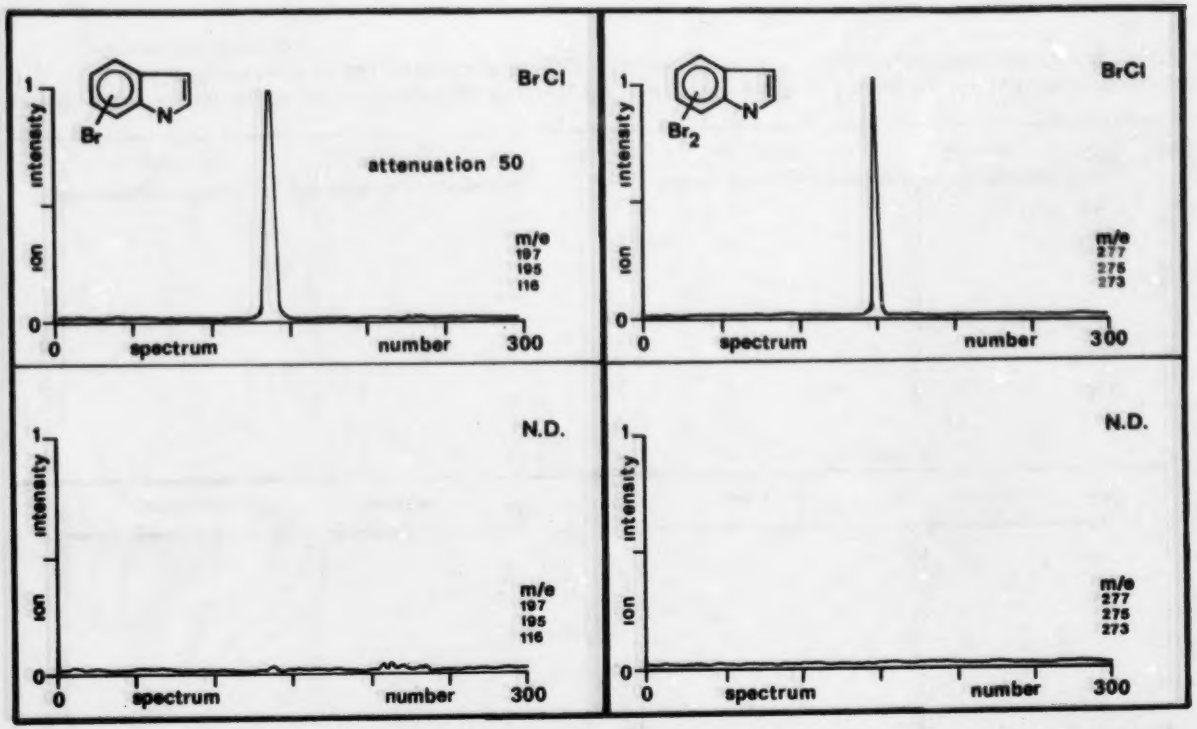

Figure 5. Selected ion summation chromatograms searching for bromoindole and dibromoindole in fathead minnows from the Wyoming, Michigan, experimental sewage treatment plant. BrCl means bromine chloride-treated. N.D. means nondisinfected.

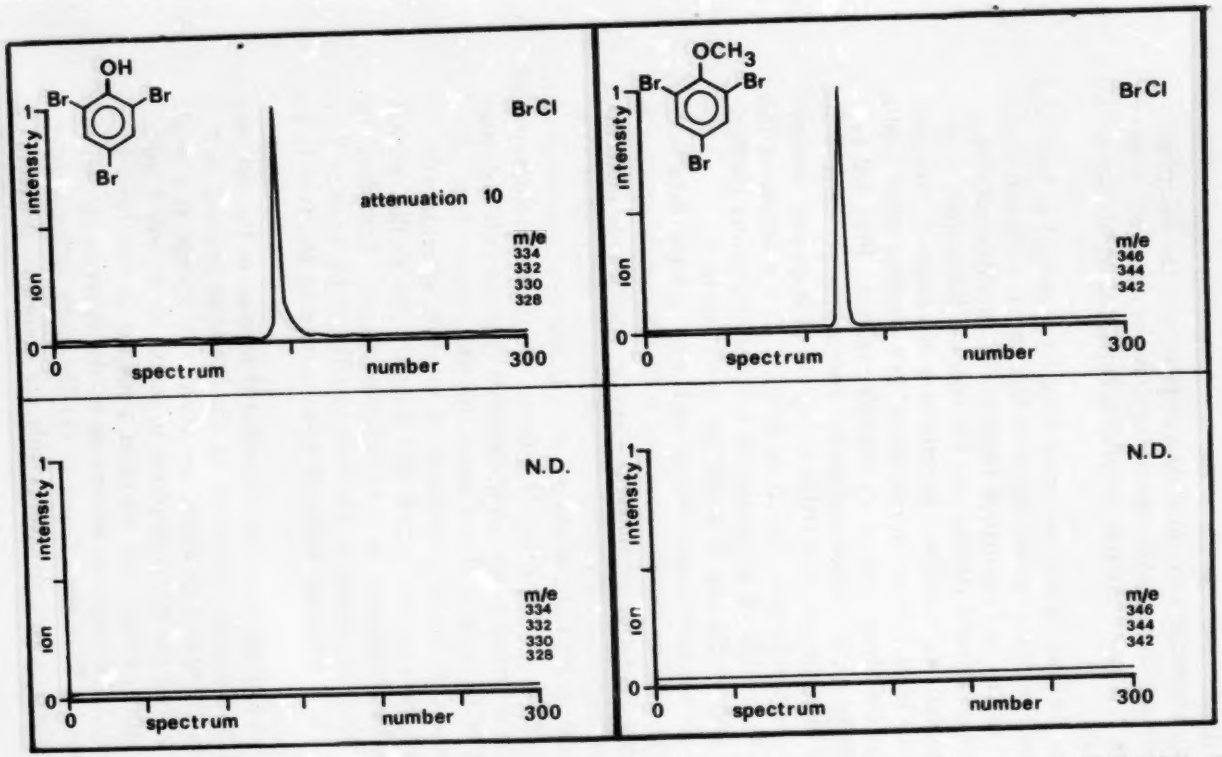

Figure 6. Selected ion summation chromatograms searching for 2,4,6-tribromophenol and 2,4,6-tribromoanisole in fathead minnows from the Wyoming, Michigan, experimental sewage treatment plant. BrCl means bromine chloride-treated. N.D. means nondisinfected.

This should have resulted in the fish from both exposures containing approximately equal concentrations of the identified brominated compounds. Since this was not observed, however, points 2, 3 and 4 are not reasonable explanations to account for the data.

The first mechanism proposed must be examined in two steps: aqueous bromination and bioaccumulation. It is known that the active species in bromination reactions by bromine water is hypobromous acid (HOBr) and the reaction mechanism is an electrophilic substitution. Bromination of phenol by bromine water to yield 2,4,6-tribromophenol[9] is a reaction often used in organic chemistry texts as an example reaction. BrCl will hydrolyze to HOBr almost exclusively over a wide pH range[10] and if any hydrobromic acid (HBr) is formed, it is quickly oxidized by hypochlorous acid (HOCl) to HOBr.[8] One example of this is the formation of a mixture of bromine-containing products when pulp is chlorine bleached using sea water.[11]

GC retention-time correlations and mass spectral data indicate that the tribromophenol identified in the Wyoming study was probably 2,4,6-tribromophenol. Similar results were obtained on a subsample of Wyoming fish which was analyzed at Dow Chemical in Midland, Michigan, by selected ion monitoring mass spectrometry.[12] At the 25% effluent level, 2,4,6-tribromophenol was not detected in the nondisinfected exposed fish and quantitated at 85 ng/g in the bromine chloride exposed fish. A 1958 study of aqueous reactions of bromine chloride with aromatic systems by Schulek and Burger[13] shows that phenol will readily brominate to 2,4,6-tribromophenol. Therefore, the bromination of phenol in the sewage effluent by bromine chloride is a potential souce of tribromophenol observed in the fish.

The identification of the brominated indoles in the fish was at first unexpected. A review of the literature showed that high concentrations of indole are present in sewage as a result of the conversion of tryptophane to indole by *E. coli*[14] and, in this study, indole was found as a residue in fish from both exposures. Although no references to the aqueous halogenation of indole by BrCl were found in the literature, other heterocyclic aromatic systems like pyrrole are known to have a

reactivity toward aqueous chlorination similar to phenols. A dinitrogen compound, antipyrine, was found to readily add bromine during aqueous chlorobromination to yield 4-bromoantipyrine[13] and chloroindole has previously been isolated in effluents after chlorination.[15] Thus, the bromination of the indoles is a potential source of the polybrominated indoles observed in the fish. The bioconcentration tests illustrated that the brominated indoles, phenols and anisoles are concentrated in the fish. The estimated residue concentrations (Tables II and III) could have arisen by bioconcentration through the gills if the water concentrations of the respective chemicals in wastes were greater than 20 μg/l for bromoindole, 1 μg/l for the tribromophenol and 0.08 μg/l for the tribromoanisole. Because the 5-bromoindole has a bioconcentration factor below 10 and residues in fish exposed to 10 μg/l in water were below detection limits, the water concentration necessary to cause 0.1-0.2 μg/g residues observed in this study could be 100 μg/l or greater.

A second possible explanation for the observed residue data was the synthesis of brominated compounds by fathead minnows. Neutron activation analysis of the Wyoming study fathead minnows indicated 71 μg/g total bromine in the bromine chloride exposed fish vs 8 μg/g in the nondisinfected fish[12] indicating that a supply of bromine is available to the fish from its own electrolyte pool. It has been shown that brominated compounds do exist in and may be synthesized by various marine algae which may account for the background levels of brominated compounds present in marine fish oils.[16] No literature references could be found to indicate that marine or freshwater fish, in particular the fathead minnow, can synthesize brominated organic chemicals. However, unpublished results of Dow Chemical[12] indicated 0.04 μg/g of tribromophenol and tribromoanisole in fathead minnows exposed to 100 mg/l bromide ion which suggests biosynthesis is possible.

SUMMARY AND CONCLUSION

Results of two studies have shown that brominated organic chemicals were found in fish exposed to wastewater disinfected

with bromine chloride, whereas the same chemicals were not detected in fish exposed to the nondisinfected effluent. The observed chemicals are apparently not a result of some outside contamination but probably arise from the bromination of precursors as a result of disinfection.

One possible mechanism to explain the observed data is the aqueous bromination of precursors followed by bioaccumulation of the products by the fish. A review of the literature indicates that aqueous bromination of organic compounds does readily occur with bromine chloride. The conclusion of one paper is that aqueous bromine chloride can be used for preparative purposes in organic chemistry or as a standard titrant in analytical chemistry.[13] Bromine chloride is a selective brominating agent for phenols and the synthesis of a series of bromophenols and bromocresols using bromine chloride has been patented.[17] Experiments with three of the identified brominated chemicals and fathead minnows have shown that these chemicals readily bioaccumulate.

Another possible mechanism to explain the observed data is the bromination of precursors by the fish themselves. Neutron activation analysis has shown that a large supply of bromine is available to the fish exposed to BrCl-treated effluent for the possible synthesis of brominated organics. However, no evidence is available to indicate that the fathead minnow can synthesize brominated chemicals.

The purpose of this chapter is not then to suggest that bromine chloride should or should not be used as an alternate disinfectant to chlorination. Rather, we have presented evidence that chlorobromination of wastewaters results in organobromine residues in fish exposed to them. Although the organobromine residues were observed at concentrations at least an order of magnitude less than the toxic chemicals such as PCB and chlordane that were present in the raw wastes, the smaller bioconcentration factors for the polar brominated chemicals suggest that measurable water concentrations of these chemicals are likely to be present in the chlorobrominated wastewater. Therefore, it appears necessary that the aqueous chemistry of bromination of organic chemicals and measurement of the products in wastewaters be studied to determine if chlorobromination

is more advantageous then chlorination with respect to the halogenated chemicals produced.

ACKNOWLEDGMENT

We wish to thank Ms. Barbara Bergsted for her assistance with the bioaccumulation study.

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